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Clinical Significance of Serum Hepcidin Levels on Early Infectious Complications in Allogeneic Hematopoietic Stem Cell Transplantation

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Short title: Significance of hepcidin in transplantation

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ABSTRACT

The association of iron overload with complications of allogeneic hematopoietic stem cell transplantation (HSCT) has been suggested in previous studies. Since hepcidin plays a central role in the regulation of iron homeostasis, we analyzed the association between pre-transplant serum hepcidin-25 levels and early infectious complications after allogeneic HSCT. We studied 55 consecutive adult patients with a median age of 47 years (range, 20–64 years) who underwent allogeneic HSCT for hematologic malignancies at our institution. Thirty-two patients had myeloid malignancies; the remaining 23 had lymphoid malignancies. The median pre-transplant serum hepcidin level of patients in the study was 21.6 ng/ml (range, 1.4–371 ng/ml), which was comparable to that of healthy volunteers (median, 19.1 ng/ml [range, 2.3–37 ng/ml]; $n = 17$). When cumulative incidences of documented bacterial and cytomegaloviral infections at day 100 were compared according to pre-transplant hepcidin-25 levels, the incidence of bacterial, but not cytomegaloviral, infection, was significantly higher in the high-hepcidin group (≥ 50 ng/ml; $n = 17$) than in the low-hepcidin group (< 50 ng/ml; $n = 38$) (65% [95% confidence interval, 38%–82%] vs 11% [3%–23%]; $P < 0.001$). This finding was confirmed by multivariate Cox analysis adjusted for confounders, including pre-transplant ferritin and C-reactive protein levels. No fungal infection was documented in either group. These results suggest that the pre-transplant serum hepcidin-25 level may be a useful marker for predicting the risk of early bacterial complications after allogeneic HSCT. Larger prospective studies are, however, warranted to confirm our findings.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) has been widely performed as a potentially curative treatment for intractable hematologic malignancies with conventional chemotherapy. However, despite recent advances in the treatment of infectious diseases and conditioning regimens for transplantation, treatment-related complications remain a major problem. Therefore, it is particularly important to identify a good biomarker that can predict treatment-related complications before transplantation. A recently accumulated body of evidence suggests that iron overload is associated with adverse clinical outcomes in HSCT [1-10]. Armand et al. showed that a high pre-transplant serum ferritin level was strongly associated with lower overall and disease-free survival in patients with allogeneic HSCT that was performed as a treatment for acute leukemia and myelodysplastic syndrome (MDS) [2]. Other studies have shown that pre-transplant iron overload in autologous or allogeneic HSCT was a risk factor associated with post-transplant complications, such as mucositis, bacterial and fungal infection, and hepatic veno-occlusive disease [3-6, 8-11].

Hepcidin, first identified in human blood and urine as an antimicrobial small peptide [12, 13], is now considered to be a central molecule that regulates iron metabolism. Hepcidin decreases iron absorption from the intestine and blocks its release from iron stores by down-regulating the expression of the cellular iron exporter, ferroportin [14, 15]. Hepatic expression of hepcidin can be up-regulated by iron loading [16, 17] as well as by inflammatory stimuli such as interleukin-6 (IL-6) [18]. Therefore, we hypothesized that

serum hepcidin level could be a useful predictor of iron overload and inflammatory condition prior to HSCT. Here, we performed a single-center retrospective study at our institution in order to evaluate the significance of serum hepcidin levels as a predictor of early treatment-related complications after allogeneic HSCT with special reference to infectious complications.

PATIENTS AND METHODS

Study Population

The study population comprised 66 consecutive adult patients who underwent allogeneic HSCT for the treatment of hematologic malignancies at Kyoto University Hospital from July 2006 to September 2008. A total of 55 patients, excluding those who had received prior transplantations within one year or who had any active infections before the current transplantation, were included in the analysis. This study was approved by the Ethics Committee of Kyoto University Graduate School and the Faculty of Medicine. Written informed consent was obtained from all patients.

Serum Analysis

Before the administration of conditioning regimens, serum samples were obtained at around 8:00 am, allocated in tubes, and stored at -80°C until analysis. The levels of serum hepcidin-25 (the main form of active hepcidin peptide) were quantified using a liquid chromatography-tandem mass spectrometry-based assay system following the method described by Murao et al. [19]. Other serum parameters were measured using standard laboratory techniques.

Prophylaxis, Monitoring, and Diagnosis of Infection

The patients were isolated in a single room equipped with a high-efficiency particulate air filter (HEPA) system from one day before transplantation until at least four weeks after transplantation. No bacterial prophylaxis was prescribed for the patients according to our institutional protocols [20]. Trimethoprim-sulfamethoxazole (160 mg/day [trimethoprim], three times a week) was administered as prophylactic therapy for *Pneumocystis jirovecii* pneumonia from the day of admission until the day of transplantation and restarted after the day of neutrophil engraftment. All patients received fluconazole (200 or 400 mg/day) and acyclovir (1000 mg/day) prophylaxis from the period of conditioning until 30 days after transplantation. After the first 30 days, the patients received fluconazole at a dose of 100 mg/day until at least 100 days after transplantation. The administration of acyclovir (400 mg/day) was continued when patients received steroid therapy for acute graft-versus-host diseases. For each febrile episode, one or two sets of blood samples were cultured, and the cultures of specimens other than blood and imaging examinations were performed according to clinical judgment. The occurrence of cytomegaloviral (CMV) infection was closely monitored by CMV pp65 antigenemia testing with C10/C11 monoclonal antibodies from the day after neutrophil engraftment until at least 100 days after transplantation. Documented bacterial infection included any incidence of bloodstream infection or any other bacterial infection. Bloodstream infection was diagnosed if at least one of the following criteria was met: (1) Blood culture obtained during a febrile episode was positive, at least once, for bacterial organisms not considered to be common skin contaminants; (2) blood culture

obtained during a febrile episode was positive for the same common skin contaminant on separate occasions within 72 h; (3) blood culture was positive, at least once, for a common skin contaminant, and the patient was diagnosed with septicemia, including hypotension (systolic blood pressure, <90 mmHg) and abnormal coagulopathy. Infections other than bloodstream infection were diagnosed if the following criteria were met: (1) Bacterial organisms were observed from specimens such as sputum, urine, and stool at least on two occasions and (2) the patient showed symptoms of infection corresponding to those specimens. *C. difficile* enterocolitis was excluded from the analysis, because this disease is toxin-mediated, and cannot be prevented by administration of common bacterial prophylactic agents such as fluoroquinolones, even if patients with a high risk of bacterial infection can be identified by using a putative biomarker. CMV infection was defined as positive if either C10 or C11 antigenemia assay showed at least 2 positive cells per 150,000 leukocytes. Invasive fungal infection was diagnosed according to the criteria of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group [21].

Statistical Analysis

Endpoints included cumulative incidences of documented bacterial infection, fungal infection, CMV infection, and infection-related mortality, and overall survival during 100 days after transplantation. Patient and transplant characteristics between two groups were

compared using the Mann-Whitney U test or χ^2 analysis, as appropriate. The day of neutrophil engraftment was defined as the first of three consecutive days when the absolute neutrophil count exceeded 500/ μ L. The day of neutrophil engraftment between two groups was compared by using the Mann-Whitney U test. To eliminate the effect of competing risk, the cumulative incidences were assessed using methods described elsewhere [22]. The competing event in the cumulative incidence analyses was defined as death without an event of interest within 100 days after transplantation. Overall survival was estimated using Kaplan-Meier methods. Infection-related death was defined as death associated with any infection during 100 days after transplantation. Standard risk disease was defined as complete remission (CR) in cases of acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), adult T cell leukemia/lymphoma (ATL), Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), and untreated or CR in MDS and myeloproliferative disorder (MPD). High risk disease was defined as statuses other than CR in AML, ALL, ATL, HL, and NHL and in MDS and MPD after treatment. The Cox proportional-hazard model was applied to assess the effect of factors that potentially affected the study endpoints. The following items were added as confounders: Recipient's gender (male or female), recipient's age (<50 or \geq 50 years), diagnosis (myeloid or lymphoid malignancies), risk of disease (standard or high risk), conditioning regimen (reduced or myeloablative intensity), type of donor (related or unrelated donor), reticulocyte count (< 60×10^9 or $\geq 60 \times 10^9$ /l), ferritin level (<1000 or \geq 1000 mg/dl), and C-reactive protein (CRP) level (<0.3 or \geq 0.3 μ g/dl). The cutoff points for reticulocyte

count and the ferritin and CRP levels were chosen such that we could make optimal use of the information with a proviso that the smaller group contained at least 30% of patients. P values of less than 0.05 were considered statistically significant. All analyses were conducted using STATA software version 10 (STATA Corp., College Station, TX, USA).

RESULTS

Characteristics of Patients and Transplants

Characteristics of patients and transplants are shown in Table 1. The median age of patients was 47 years (range, 20–64 years). The primary disease in these patients was as follows: AML in 23, MDS/MPD in 9, ALL in 8, NHL in 9, HL in 1, and ATL in 5. The risk of diseases was standard in 27 and high in 28 patients. Nearly half of the patients ($n = 26$) received a reduced-intensity conditioning regimen. The stem cell sources used were bone marrow in 39, peripheral blood in 1, and cord blood in 15 patients. The median pre-transplant serum hepcidin level was 21.6 ng/ml (range, 1.4–371 ng/ml), which was comparable to that of healthy volunteers (median, 19.1 ng/ml [range, 2.3–37 ng/ml]; $n = 17$) [23]. Since the lower hepcidin level of the 3rd tertile among the patients in this study was 49.1 ng/ml, we set a cutoff hepcidin level of 50 ng/ml for practical use to divide the patients into low- and high-hepcidin groups ($n = 17$ and 38, respectively). There was no difference in patient and transplant characteristics between the low- and high-hepcidin groups, except for serum ferritin levels ($P < 0.001$).

Documented Bacterial Infections

There was no significant difference between the days of neutrophil engraftment of the low- and high-hepcidin groups (median day, 21 [range, 14–99] and median day, 22.5 [range, 12–53], respectively, $P = 0.54$). A total of 16 episodes of bacterial infections were

documented; these included 15 episodes of bloodstream infections and 1 episode of pneumonia. No patient experienced more than one episode of bacterial infection within 100 days after transplantation. The documented bacterial organisms are listed in Table 2. The main organisms were gram-negative bacilli in both the low- and high-hepcidin groups. In the antimicrobial-susceptibility tests, 12 out of the 13 gram-negative isolates were sensitive to fluoroquinolone. We documented two bacterial infections in the late period of transplantation; one patient showed infection at day 89 after transplantation, which was attributed to delayed neutrophil engraftment, and another patient showed infection at day 68, when the neutrophil counts had temporarily decreased. The cumulative incidences of the documented bacterial infection in the low- and high-hepcidin groups were 11% (95% confidence interval [CI], 3%–23%) and 65% (95% CI, 38%–82%), respectively (Figure 1A). In the low-hepcidin group, the cumulative incidence of bacterial infection was lower in patients with a hepcidin level of <25 ng/ml than in those with a hepcidin level ranging from ≥ 25 to <50 ng/ml (10% [95% CI, 2%–23%] vs 17%, [95% CI, 1%–52%]). Univariate analysis of various potential confounders showed that high hepcidin level was the only factor that affected the cumulative incidence of documented bacterial infection (hazard ratio [HR], 8.98; 95% CI, 2.82–28.57; $P < 0.001$) (Table 3). To exclude the effect of other confounders, the significance of high hepcidin level was assessed in the stratified category of each confounder (e.g., in either the high- or low-ferritin group); we noted consistently high HRs in the high-hepcidin group in each stratified category (data not shown). We also found that

hepcidin had a significant impact on the patients, excluding the patients in other specific categories, such as those who received a cord-blood transplant or those who underwent a transplant from an unrelated HLA-mismatched donor (data not shown). Furthermore, the significant effect of hepcidin persisted even after the adjustment for confounders in multivariate analysis (HR, 28.46; 95% CI, 2.51–323.34; $P = 0.007$) (Table 3). Even when the variables were treated as continuous instead of categorical, the significant effect of hepcidin persisted (HR, 1.01; 95% CI, 1.00–1.01; $P = 0.001$).

Other Transplant-related Complications and Mortality

The cumulative incidences of CMV infection in the low- and high-hepcidin group were 49% (95% CI, 32%–64%) and 45% (95% CI, 20%–67%), respectively (Figure 1B); univariate and multivariate analyses showed no significant difference between the two groups (Table 3). All CMV infections were well treated by the administration of ganciclovir or foscarnet. No fungal infection was documented. Therefore, all infection-related deaths were attributed to bacterial infection. The cumulative incidence of infection-related mortality in the low-hepcidin group was 3% (95% CI, 0.2%–12%), while that in the high-hepcidin group was 6% (95% CI, 0.4%–24%), with no statistical difference between two groups. Overall survival at 100 days after transplantation in the low- and high-hepcidin groups was 95% (95% CI, 81%–99%) and 82% (95% CI, 55%–94%), respectively (Figure 2). No significant difference in overall survival was observed (Table 3).

DISCUSSION

In our cohort of patients who underwent allogeneic HSCT for hematologic malignancies, we found a significant association between the pre-transplant serum hepcidin levels and the cumulative incidence of documented bacterial infection. To our knowledge, this is the first study that has evaluated the clinical significance of serum hepcidin levels in predicting transplant-related complications; the findings suggest that the pre-transplant serum hepcidin level can be used as a good pre-transplant biomarker to predict bacterial infection in a patient scheduled for HSCT.

Hepcidin production is regulated by at least three factors: Iron load [16, 17], inflammation [18], and unknown erythropoietic signals [23-25]. Therefore, the good predictive value of hepcidin with respect to the incidence of documented bacterial infection can be partly explained by the cumulative effect of at least these three factors on bacterial infection. Iron overload increases the level of circulating non-transferrin-bound iron, which is known to amplify free-radical reactions in inflammatory or ischemia-related conditions [7, 26]. Such reactions could enhance tissue damage such as mucositis during the conditioning regimen, thereby allowing bacterial translocation through the damaged mucosa [27]. In addition, iron is a necessary nutrient for bacteria and fungus [28]. The association between hemochromatosis, one of the iron overload disorders, and infection with certain organisms has already been described [29]. Therefore, the high hepcidin levels might reflect iron

overload status, which has an adverse effect on bacterial infections. Second, a high hepcidin level may indicate inflammation due to a latent bacterial infection that was undetectable before HSCT but may surface in post-transplant neutropenic status. Lastly, a high hepcidin level could reflect suppressed erythropoiesis, probably due to the short duration from the last chemotherapy to the start of the conditioning regimen for transplantation. Repeated cytotoxic chemotherapy in a short period may exacerbate tissue damage and increase the risk of bacterial infection.

Although serum ferritin levels do not necessarily correlate with the amount of iron load in patients with inflammation or specific diseases [1, 30, 31], it is frequently used and regarded as a indicator of iron overloading, and several studies have demonstrated the association between high ferritin levels and treatment-related mortality [3, 11]. In this cohort, an elevation of serum ferritin level was not found to be a significant risk factor for bacterial infection, while an elevated hepcidin level was a strong risk factor even after adjustment for other potential confounders. Furthermore, we observed consistent association of high hepcidin levels with high risk for developing bacterial infection when analyses were confined to either the low- or high-ferritin subgroups. These findings collectively suggest that hepcidin can be used as a better predictor of documented bacterial infections than serum ferritin levels. Moreover, various new techniques to quantify hepcidin-25, such as a competitive enzyme-linked immunoassay as well as mass spectrometry-based methods, have been

recently developed [19, 25, 32, 33]. Standardization of those methods will make it possible to use the serum hepcidin level as a biomarker in routine clinical practice.

Hepcidin was first isolated and characterized as an antimicrobial peptide in human blood [12]. In radial diffusion assays, synthetic hepcidin suppressed the growth of several strains of gram-positive bacteria and some strains of gram-negative bacteria, but not of *Escherichia coli* or *Pseudomonas fluorescens*. Our findings pertaining to the adverse association of high hepcidin levels with bacterial infection indicated that the bactericidal effect of hepcidin was either considerably limited in neutropenic settings such as HSCT or was ineffective on the bacterial organisms observed in our cohort. Moreover, we observed a significant adverse effect of hepcidin even after the adjustment for potential confounders, suggesting that hepcidin itself may play an unknown biological role in susceptibility to bacterial infection, or it may represent another unknown surrogate marker for predicting bacterial infection. To answer this issue, the significance of pre-transplant serum hepcidin levels needs to be evaluated in a more homogeneous group of patients having the same level of confounders.

We did not detect any adverse effect of high hepcidin levels on infection-related mortality or overall survival at 100 days after transplantation, although there was a marked difference in the incidence of bacterial infection. One possible explanation for this

observation is that bacterial infection of the blood was well managed by prompt and appropriate treatment with antibiotics in our transplant centers. However, because the incidence of early death after HSCT is considerably low, the effect of bacterial infection on early mortality should be evaluated in larger cohort studies in order to gain enough statistical power for comparison. Alternatively, selective prophylactic administration of oral antibiotics such as fluoroquinolones to patients with a high risk of bacterial infection may be an effective approach; however, this approach will be effective only if most of the bacterial isolates at the transplant center are sufficiently sensitive to these prophylactic antibiotics. With regard to other endpoints, there was no association between high hepcidin levels and the incidence of CMV infection. The effect of hepcidin level on the incidence of fungal infection could not be evaluated because of the very low incidences of these conditions in our cohorts. These effects should also be evaluated in studies with a larger cohort in the future.

The present study, however, has some limitations. We cannot exclude the possibility of a pseudo-negative result for bloodstream infection, because broad-spectrum antibiotics were administered to all neutropenic patients at the time of blood culture, regardless of the results of blood culture. In addition, the retrospective study design and heterogeneous background of diseases and transplantation procedures could also bias the results. Particularly, in the small cohort of 55 patients, the adjustment of HRs by confounders may be incomplete. In particular, the higher proportion of cord-blood transplants and the high risk of diseases in the

high-hepcidin group may cause bias, although we found consistently high HRs in the high-hepcidin group in various stratified categories. Therefore, larger studies are necessary to confirm our results.

In conclusion, our study revealed that the pre-transplant serum hepcidin level was significantly associated with bacterial infection, particularly bloodstream infection, suggesting that quantification of serum hepcidin levels could be useful for predicting early bacterial complications. Prophylactic antibiotic therapy based on the local sensitivities of common bacterial isolates can be considered in the patients with high hepcidin levels who are undergoing allogeneic HSCT. Larger prospective studies are, however, warranted to confirm our findings.

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Conflict of interest: NT declares that he is the President of Medical Care Proteomics Biotechnology Co. Ltd. (Ishikawa-ken, Japan), a start-up company, the stock of which is not publicly traded. The other authors declare that they have no conflicts of interest relevant to this paper.

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TABLES

Table 1. Characteristics of Patients and Transplants

Variables	Hepcidin, low (<50 ng/ml) n = 38	Hepcidin, high (≥50 ng/ml) n = 17	P Value
Age at transplant			
Median age (range)	47.5 (23–64)	47 (20–63)	0.750
Sex			0.171
Male	21 (55%)	6 (35%)	
Female	17 (45%)	11 (65%)	
Disease			0.612
Myeloid malignancies	23 (61%)	9 (53%)	
AML	15	8	
MDS/MPD	8	1	
Lymphoid malignancies	15 (39%)	8 (47%)	
ALL	4	4	
ATL	4	1	
HL	1	0	
NHL	6	3	
Risk of disease			0.051
Standard	22 (58%)	5 (29%)	
High	16 (42%)	12 (71%)	
Conditioning regimen			0.545
Myeloablative intensity	19 (50%)	10 (59%)	
Reduced intensity	19 (50%)	7 (41%)	
Prophylaxis against GVHD			0.663
Cyclosporine-based	5 (13%)	3 (18%)	
Tacrolimus-based	33 (87%)	14 (82%)	
Type of donor			0.181
Related donor			
HLA*-matched	10 (26%)	3 (18%)	
HLA-mismatched	3 (8%)	1 (6%)	
Unrelated donor			

HLA-matched	18 (47%)	5 (29%)	
HLA-mismatched	7 (18%)	8 (47%)	
Source of stem cells			0.259
Bone marrow	29 (76%)	10 (59%)	
Peripheral blood	1 (3%)	0 (0%)	
Cord blood	8 (21%)	7 (41%)	
Serum ferritin (μg/dl)			
mean (± SD)	664 (± 796)	1551 (± 993)	<0.001
CRP (mg/dl)			
mean (± SD)	0.36 (± 0.68)	0.70 (± 1.63)	0.176
Reticulocyte (×10⁹/l)			
mean (± SD)	63.7 (± 40.2)	64.0 (± 42.2)	0.979

Data are counts of individuals unless specified otherwise. AML indicates acute myelogenous leukemia; MDS/MPD, myelodysplastic syndrome and myeloproliferative disorders; ALL, acute lymphoblastic leukemia; ATL, acute T cell leukemia/lymphoma; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; Cyclosporine-based, cyclosporine with or without other agents; Tacrolimus-based, tacrolimus with or without other agents; HLA, human leukocyte antigen; CRP, C-reactive protein.

*HLA compatibility was defined according to the results of serologic or low-resolution molecular typing for HLA-A, B, and DR antigens.

Table 2. Documented Bacterial Organisms within 100 Days after Stem Cell Transplantations

Category	Hepcidin, low (<50 ng/ml) n = 38	Hepcidin, high (≥50 ng/ml) n = 17
Gram-positive cocci (n)	<i>Staphylococcus epidermidis</i> (1)	<i>Enterococcus faecium</i> (2)
Gram-negative bacilli (n)	<i>Klebsiella pneumoniae</i> (2) <i>Enterobacter cloacae</i> (1) <i>Prevotella intermedia</i> (1)	<i>Klebsiella pneumoniae</i> (3) <i>Escherichia coli</i> (3) <i>Pseudomonas aeruginosa</i> (2) <i>Klebsiella oxytoca</i> (1)

P. intermedia was detected in the sputum of one patient with pneumonia. Other organisms were detected in blood culture bottles.

Table 3. Univariate and Multivariate Analyses of Documented Bacterial Infection, CMV Infection, and Overall Survival at 100 Days after Stem Cell Transplantations

	Number	Univariate Analysis		Multivariate Analysis	
		HR (95% CI)	<i>P</i> value	HR (95% CI)	<i>P</i> value
1) Documented bacterial infection					
Hepcidin, low (<50 ng/ml)	5/38	1	–	1	–
Hepcidin, high (≥50 ng/ml)	11/17	8.98 (2.82–28.57)	<0.001	28.46 (2.51–323.34)	0.007
2) CMV antigenemia (C10 or C11 ≥2)					
Hepcidin, low (<50 ng/ml)	18/37	1	–	1	–
Hepcidin, high (≥50 ng/ml)	7/16	0.97 (0.40–2.32)	0.939	0.63 (0.16–2.49)	0.511
3) Overall survival					
Hepcidin, low (<50 ng/ml)	36/38	1	–	–	–
Hepcidin, high (≥50 ng/ml)	14/17	3.60 (0.60–21.56)	0.161	–	–

CMV indicates cytomegalovirus; CI, confidence interval. Hazard ratios (HRs) in multivariate analysis were adjusted for recipient's gender (male or female), recipient's age (<50 or ≥50 years), diagnosis (myeloid or lymphoid malignancies), risk of disease (standard or high risk), conditioning regimen (reduced or myeloablative intensity), type of donor (related or unrelated donor), reticulocyte count ($<60 \times 10^9$ or $\geq 60 \times 10^9$ /l), ferritin level (<1000 or ≥1000 mg/dl), and C-reactive protein (CRP) level (<0.3 or ≥0.3 μg/dl). Overall survival was not analyzed in the multivariate model because of the low incidence of death.

FIGURE LEGENDS

Figure 1.

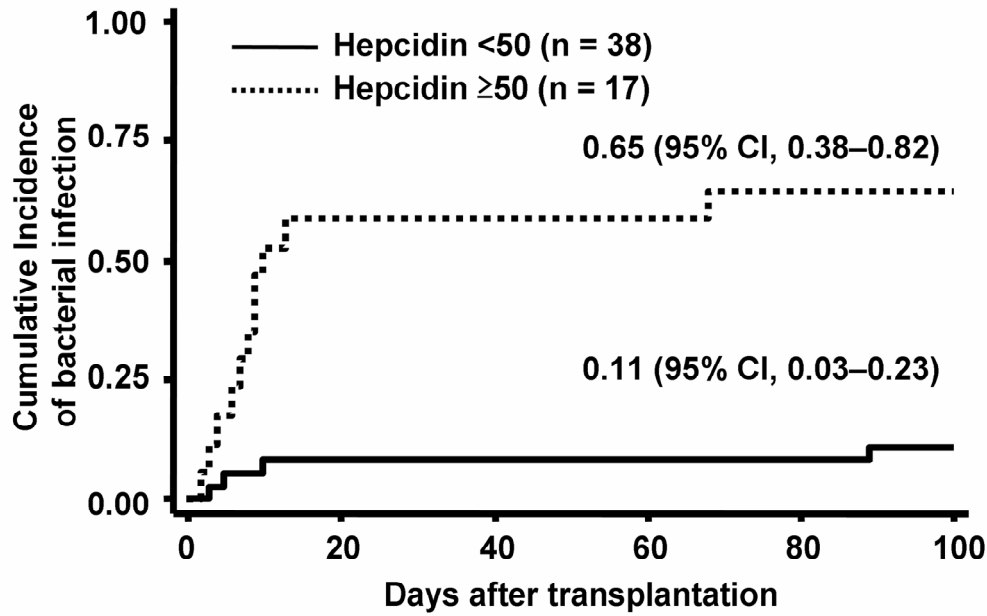
The cumulative incidences of documented bacterial infection (A) and cytomegaloviral (CMV) infection (B) at 100 days after stem cell transplantation. Solid black line, the low-hepcidin group (<50 ng/ml); solid gray line, the high-hepcidin group (≥ 50 ng/ml); CI, confidence interval. CMV infection was not assessable in two patients due to early death before neutrophil engraftment.

Figure 2.

Kaplan-Meier estimate of overall survival at 100 days after stem cell transplantation. Solid black line, the low-hepcidin group (<50 ng/ml); solid gray line, the high-hepcidin group (≥ 50 ng/ml); CI, confidence interval.

Figure 1.

A)



B)

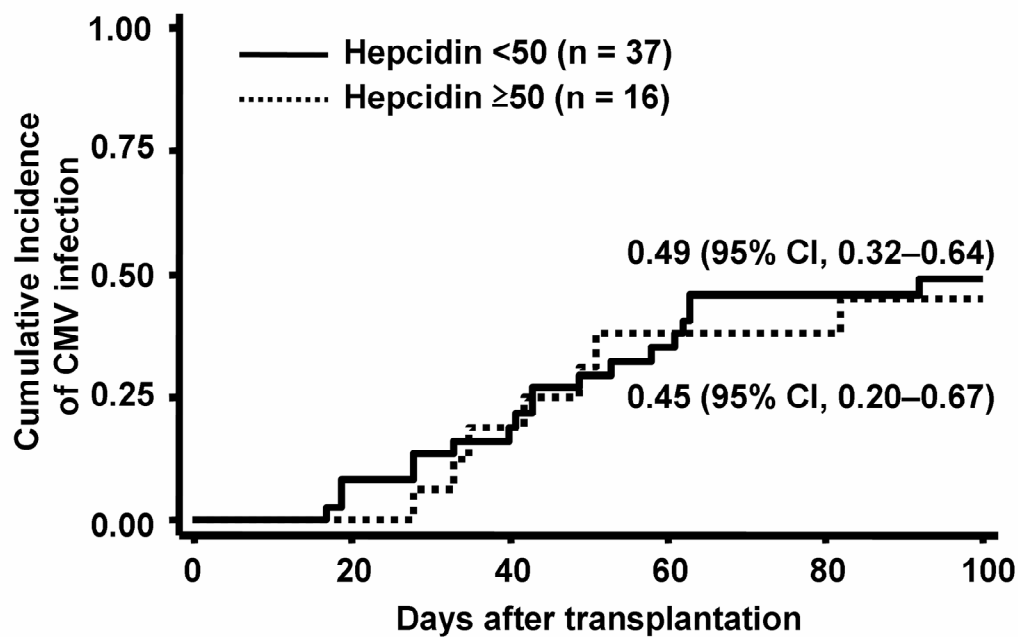


Figure 2.

